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Antitumor Efficacy of a Novel Class of Non-thiol-containing Peptidomimetic Inhibitors of Farnesyltransferase and Geranylgeranyltransferase I: Combination Therapy with the Cytotoxic Agents Cisplatin, Taxol, and Gemcitabine[1]

Jiazhi Sun, Michelle A. Blaskovich, David Knowles, Yimin Qian, Junko Ohkanda, Ratna D. Bailey, Andrew D. Hamilton, and Saïd M. Sebti[2]

Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, Florida 33612 [J. S., M. A. B., R. D. B., S. M. S.], and Yale University, Department of Chemistry, New Haven, Connecticut 06511 [D. K., Y. Q., J. O., A. D. H.]

ABSTRACT

Ras malignant transformation requires posttranslational modification by farnesyltransferase (FTase). Here we report on the design and antitumor activity, in monotherapy as well as in combination therapy with cytotoxic agents, of a novel class of non-thiol-containing peptidomimetic inhibitors of FTase and the closely related family member geranylgeranyltransferase I (GGTase I). The non-thiol-containing FTI-2148 is highly selective for FTase (IC_{50}, 1.4 nM) over GGTase I (IC_{50}, 1700 nM), whereas GGTI-2154 is highly selective for GGTase I (21 nM) over FTase (IC_{50}, 5600 nM). In whole cells, the corresponding methylester prodrug FTI-2153 is >3000-fold more potent at inhibiting H-Ras (IC_{50}, 10 nM) than Rap1A processing, whereas GGTI-2166 is over 100-fold more selective at inhibiting Rap1A (IC_{50}, 300 nM) over H-Ras processing. Furthermore, FTI-2153 was highly effective at suppressing oncogenic H-Ras constitutive activation of mitogen-activated protein kinase and human tumor growth in soft agar. FTI-2148 suppressed the growth of the human lung adenocarcinoma A-549 cells in nude mice by 33, 67, and 91% in a dose-dependent manner. Combination therapy of FTI-2148 with either cisplatin, gemcitabine, or Taxol resulted in a greater antitumor efficacy than monotherapy. GGTI-2154 in similar antitumor efficacy experiments is less potent than FTI-2148 and inhibits tumor growth by 9, 27, and 46%. Combination therapy of GGTI-2154 with cisplatin, gemcitabine, or Taxol is also more effective. Finally, FTI-2148 and GGTI-2154 are 30- and 33-fold more selective and 30- and 16-fold more potent in whole cells than our previously reported thiol-containing FTI-276 and GGTI-297, respectively. Thus, our results demonstrate that this highly potent and selective novel class of non-thiol-containing peptidomimetics inhibits human tumor growth in whole animals and that combination therapy with cytotoxic agents is more beneficial than monotherapy.

INTRODUCTION

The ability of oncogenic Ras, a low molecular weight GTPase, to induce malignant transformation requires lipid modification by a farnesyl group. This prompted us and others to design, synthesize, and biologically evaluate inhibitors of FTase[3] as potential anticancer drugs (1, 2). FTase is an $\alpha\beta$ heterodimer enzyme that catalyzes the transfer of farnesyl from farnesyl PP_i to cysteines in the fourth position from the COOH terminal of proteins that end with the consensus sequence CAAX (C is cysteine, A is aliphatic, and X is any residue but leucine or isoleucine; Refs. 3 and 4). Proteins that end with CAAX where X is leucine or isoleucine are posttranslationally modified by a closely related enzyme, GGTase I, that shares the α subunit with FTase (4). We have made CAAX tetrapeptide mimetics that are potent and selective for FTase and GGTase I (1). For example, FTI-276 and GGTI-297 are CAAM and CAAL peptidomimetics where the dipeptide "AA" was replaced by 2-phenyl-4-aminobenzoic acid and 2-naphthyl-4-aminobenzoic acid, respectively (5, 6). FTI-276 inhibits selectively FTase (IC_{50}, 0.5 nM) over GGTase I (IC_{50}, 50 nM), whereas GGTI-297 inhibits selectively GGTase I (IC_{50}, 55 nM) over FTase (IC_{50}, 203 nM). FTI-277 and GGTI-298, the methylester prodrugs of FTI-276 and GGTI-297, respectively, affect cellular physiology quite differently. Although both FTI-277 and GGTI-298 inhibit the growth of cultured human tumor cells, GGTI-298 blocks all tumor types in the G_1 phase of the cell cycle, whereas FTI-277 either blocks in G_1, enriches in G_2-M, or has no effect on cell cycle distribution, depending on the tumor cell line (7, 8). Furthermore, in fibroblasts, GGTI-298 but not FTI-277 blocks platelet-derived growth factor-stimulated receptor tyrosine phosphorylation (6). In smooth muscle cells, GGTI-298 enhances, whereas FTI-277 blocks, the ability of interleukin 1β to induce nitric oxide synthase 2 (9). Finally, in both smooth muscle cells and a human lung adenocarcinoma (A-549), GGTI-298, but not FTI-277, induces programmed cell death (apoptosis; Refs. 10 and 11). Although these results clearly indicate that inhibition of protein farnesylation has different consequences than inhibition of protein geranylgeranylation, recently we have demonstrated that both FTI-276 and GGTI-297 inhibit the growth of human tumors in nude mice, but that FTI-276 was more effective (12). Although we (12, 13) and others (14–16) have demonstrated in animal models that FTIs inhibit human tumor growth, it is not known whether inhibition of protein farnesylation alone will be sufficient to suppress human tumor growth in patients. Recent reports have demonstrated that FTIs can induce apoptosis but only when cells were either prevented from attaching to the substratum or deprived of growth factors (17, 18). Furthermore, because of the heterogeneous nature of tumor cells, drug resistance to FTIs could become a serious obstacle to effective treatment (19, 20). Therefore, it is important to determine whether combination therapy with agents with different mechanisms of action is beneficial. In this report, we explore this possibility with three clinically used anticancer drugs. Using cisplatin, Taxol, and gemcitabine, in combination with a novel class of non-thiol-containing, highly potent, and selective FTIs and GGTIs, we have demonstrated that combination therapy is more beneficial than monotherapy.

MATERIALS AND METHODS

Synthesis of CAAX Peptidomimetics. The thiol-containing inhibitors FTI-276, FTI-277, GGTI-297, and GGTI-298 were prepared as reported previously (21). The non-thiol derivatives FTI-2148 and FTI-2153 were prepared by the reductive amination of the methionine methyl ester adduct of 2-(2-methylphenyl)-4-aminomethylbenzoic acid with 1-trityl1–4-formyl-imidazole, followed by deprotection and deesterification (LiOH) in the case of FTI-2148.

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[2] To whom requests for reprints should be addressed, at Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612. Phone: (813) 979-6734; Fax (813) 979-6748; E-mail: sebti@moffitt.usf.edu.

[3] The abbreviations used are: FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; FTI, FTase inhibitor; GGTI, GGTase I inhibitor; MAPK, mitogen-activated protein kinase; mpk, mg per kg; q4d, every 4 days.

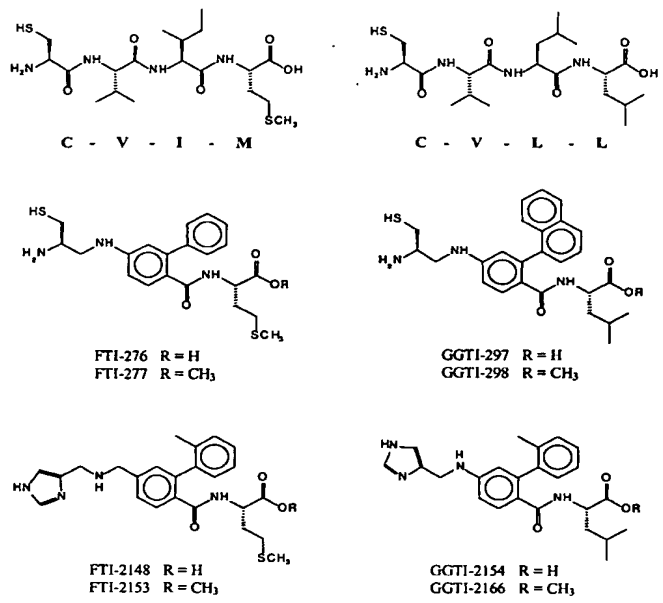

Fig. 1. Structure of thiol and non-thiol-containing CAAM and CAAL peptidomimetics.

A similar route was used with GGTI-2154 and GGTI-2166, except that reductive amination involved the leucine methyl ester adduct of 2-(2-methylphenyl)-4 aminobenzoic acid. Full details of the syntheses of these derivatives will be reported elsewhere.[4]

Cells and Materials. NIH-3T3 cells transfected with oncogenic (61L) H-Ras were obtained from Drs. Channing Der and Adrienne Cox (University of North Carolina at Chapel Hill; Ref. 22). Cells were grown in DMEM and 10% calf serum. Human tumor cell lines A-549 (lung adenocarcinoma), Panc-1 (pancreatic carcinoma), and U-87MG and DaOY (brain gliomas) were purchased from American Type Culture Collection (Rockville, MD) and grown in Ham's F-12K Nutrient Mix (A-549), DMEM (Panc-1), and MEM (U-87MG and DaOY), each containing 10% FBS, at 37°C in a humidified incubator containing 10% CO_2.

***In Vitro* FTase and GGTase I Activity Assay.** FTase and GGTase I activities from 60,000 × *g* supernatants of human Burkitt's lymphoma (Daudi) cells (American Type Culture Collection, Rockville, MD) were assayed exactly as described previously (5, 6, 13). Inhibition studies were performed by determining the ability of Ras CAAX peptidomimetics to inhibit the transfer of [^{3}H]farnesyl and [^{3}H]geranylgeranyl from [^{3}H]farnesyl PP_i and [^{3}H]geranylgeranyl PP_i to H-Ras-CVLS and H-Ras-CVLL, respectively (5, 6, 13).

Ras and Rap1A Processing Assay. Oncogenic H-Ras transformed NIH-3T3 cells were plated in DMEM and 10% calf serum on day one and treated on days 2 and 3 with either vehicle or FTI-277, FTI-2153, GGTI-298, and GGTI-2166. The cells were then harvested on day 4 and lysed in lysis buffer [30 mM HEPES (pH 7.5), 1% Triton-X-100, 10% glycerol, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 2 mM Na_3VO_4, 10 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 6.4 mg/ml 2-nitrophenylphosphate]. The lysates were electrophoresed on a 12.5% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted, respectively, with anti-Ras pan antibody (Y13–238) or an anti-Rap1A antibody (Rap1/Krev-1, Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (5, 13).

MAP Kinase Assay. Cells were treated with FTI-2153 and FTI-277 and lysed as described above for Ras and Rap1A processing. Equal amounts of lysates were separated on a 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using a phospho Erk1/Erk2-specific antibody [phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) antibody; New England Biolabs, Beverly, MA]. Antibody reactions were visualized using peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (5, 13).

Anchorage-independent Growth Assays. For soft agar growth assays, the cell lines were seeded at various cell densities in triplicate (a range of 2500 cells/well to 25,000 cells/well, depending on the cell line) in 12-well culture dishes in 0.3% agar over a 0.6% agar layer as described previously (23). Various concentrations of FTI-2153, FTI-277, or vehicle (10 mM DTT in DMSO) were included in the 0.3% agar layer of cells. Cultures were fed and treated with drug or vehicle once weekly until colonies grew to a suitable size for observation (colony growth rates were ~14–16 days for Panc-1 and Colo-357 cells and 3–4 weeks for DaOY and U87MG cells). Colonies were photographed after overnight incubation with 1 mg/ml MTT in the respective cell growth media. The growth of colonies in the presence of inhibitor was compared with the control colonies treated with vehicle (23).

Antitumor Activity in the Nude Mouse Tumor Xenograft Model. Nude mice (Charles River, Wilmington, Massachusetts) were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. A-549 cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks (7×10^6 cells/flank) of 8-week-old female nude mice, as reported previously (12, 13). When tumors reached 50–100 mm^3, animals either were dosed i.p. with 0.2 ml once daily or implanted s.c. with 2-week osmotic mini-pumps (Alzet 2002; Alza, Palo Alto, CA). When mini-pumps were used, we implanted the mini-pump on the right flank and the tumor cells on the left flank. Control animals received a saline vehicle, whereas treated animals were injected with either FTI-2148 or GGTI-2154. For combination therapy, 3 days after mini-pump implantation, control animals were dosed i.p. with 0.2 ml of vehicle saline, and treated groups were dosed i.p. with cisplatin (5 mpk, q4d, three times), gemcitabine (80 mpk, q4d, three times), and Taxol (12.5 mpk, q4d, three times). The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2)/2$) as described previously (12, 13).

RESULTS

Design of Non-Thiol-containing CAAX Peptidomimetic. Our previous efforts to design CAAX peptidomimetics as inhibitors of FTase and GGTase I have focused on replacing the central hydrophobic dipeptide "AA" or the "AAX" tripeptide by various peptidomimetics. For example, we have made FTI-276, an "AA" lacking CAAM peptidomimetic, where reduced cysteine and methionine are linked by 2-phenyl-4-aminobenzoic acid (Ref. 5; Fig. 1). Similarly, GGTI-297 is a CAAL peptidomimetic where reduced cysteine is linked to leucine by 2-naphthyl-4-aminobenzoic acid (6). FTI-265, on the other hand, is an "AAX"-lacking CAAX peptidomimetic where reduced cysteine is linked to a 3-carboxyl-biphenyl group (24). For this study, we report on the design and biological properties of non-thiol-containing CAAX peptidomimetics where the tripeptide "CAA" was replaced by an imidazole derivative of 2-(2-methylphenyl)-4-aminomethylbenzoic acid (Fig. 1). The advantage of these modifications is that the imidazole provides an oxidatively stable group that retains strong binding to zinc in the active site of FTase. The 2-methyl substituent both increases the hydrophobicity of the phenyl group and locks it into a perpendicular orientation relative to the aminomethylbenzoic acid spacer (25).

FTI-2148/2153 and GGTI-2154/2166 Are Highly Potent and Selective FTase and GGTase I Inhibitors, Respectively, *in Vitro* and in Whole Cells. To determine the potency and selectivity of FTI-2148 and GGTI-2154, we evaluated their ability to inhibit the transfer of farnesyl and geranylgeranyl from [^{3}H]FPP and [^{3}H]GGPP to H-Ras CVLS and H-Ras CVLL, respectively, as described in "Materials and Methods." Table 1 shows IC_{50}s of the inhibitors. FTI-276 (IC_{50}, 0.5 nM) is three times more potent than FTI-2148 (IC_{50}, 1.4 nM), and GGTI-2154 (IC_{50}, 21 nM) is 2.5 times more potent than GGTI-297 (IC_{50}, 55 nM). Table 1 also shows that both FTI-276 (100-fold) and FTI-2148 (1200-fold) are highly selective for FTase over GGTase I, whereas only GGTI-2154 (266-fold) and not GGTI-

[4] D. Knowles, Y. Qian, J. Marfurt, M. A. Blaskovich, S. M. Sebti, and A. D. Hamilton, manuscript in preparation.

Table 1 *Inhibition IC_{50}s for FTIs and GGTIs*

Peptidomimetics (Free acids/methylesters)	*In vitro* activity (free acids)			Whole-cell processing (methylesters)		
	FTase (nM)	GGTase I (nM)	Selectivity[a]	Ras (μM)	Rap1 (μM)	Selectivity[a]
FTI-276/FTI-277	0.5	50	100	0.3	>30	>100
FTI-2148/FTI-2153	1.4	1700	1200	0.01	>30	>3000
GGTI-297/GGTI-298	203	55	4	>15	5	>3
GGTI-2154/GGTI-2166	5600	21	266	>30	0.3	>100

[a] Fold.

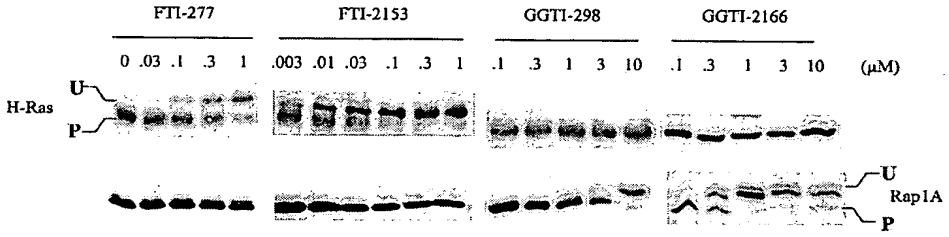

Fig. 2. Inhibition of H-Ras and Rap1A processing by FTIs and GGTIs. Oncogenic H-Ras-transformed NIH-3T3 cells were treated with the indicated concentrations of FTI-277, FTI-2153, GGTI-298, and GGTI-2166, harvested and processed for SDS-PAGE, and immunoblotted with either Ras or Rap1A antibodies as described in "Materials and Methods." *U* and *P*, unprocessed and processed proteins, respectively. Data are representative of at least three independent experiments.

297 (4-fold) is highly selective for GGTase I over FTase. Thus, for FTIs, replacement of reduced cysteine by the imidazole derivative and the phenyl substituent by tolyl (Fig. 1) resulted in much higher selectivity with little loss of potency. Similarly, for GGTIs, replacement of reduced cysteine by the imidazole derivative and the naphthyl group by a tolyl (Fig. 1) resulted in a much higher selectivity.

We next evaluated the ability of the corresponding methyl ester prodrugs to inhibit protein farnesylation and geranylgeranylation in whole cells by determining their ability to inhibit H-Ras and Rap1A processing in NIH-3T3 cells as described in "Materials and Methods." Fig. 2 shows that FTI-277 and FTI-2153 inhibited H-Ras processing with IC_{50}s of 300 and 10 nM, respectively. This inhibition is highly selective (>100 for FTI-277 and >3000 for FTI-2153) for farnesylation because the IC_{50}s for the processing of Rap1A, a geranylgeranylated protein, was higher than 30 μM (Table 1). Fig. 2 also shows that GGTI-298 and GGTI-2166 inhibited Rap1A processing with IC_{50}s of 5 μM and 300 nM, respectively. The selectivity of GGTI-298 and GGTI-2166 for inhibition of Rap1A over H-Ras processing was greater than 3-fold and 100-fold, respectively (Table 1). Thus, although *in vitro* FTI-276 is 3-fold more potent than FTI-2148, in whole cells, FTI-2153 is 30-fold more potent as well as 30-fold more selective than FTI-277. GGTI-2154 and GGTI-2166 were more potent than GGTI-297 and GGTI-298 *in vitro* and in whole cells by 2.5- and 16.7-fold, respectively. Furthermore, GGTI-2166 is much more selective (>100-fold) than GGTI-298 (>3-fold; Table 1).

FTI-2153 Is a Highly Potent Antagonist of Oncogenic H-Ras Signaling. We next determined the ability of FTI-2153 to inhibit oncogenic H-Ras signaling in NIH-3T3 cells transformed by a GTP-locked 61-leucine mutant of H-Ras. Because MAPK is a major downstream mediator of oncogenic H-Ras signaling, we investigated the effects of FTI-2153 on oncogenic H-Ras activation of MAPK. H-Ras-transformed NIH-3T3 cells were treated with various concentrations of either FTI-277 or FTI-2153 for 48 h and lysed, and the lysates were immunoblotted with an antibody that recognizes specifically the phosphorylated forms of erk1 and erk2 as described in "Materials and Methods." Fig. 3 shows that in the absence of inhibitor treatment, H-Ras-transformed NIH-3T3 cells contained higher levels of activated hyperphosphorylated erk1 and erk2 than NIH-3T3 cells transfected with the corresponding empty vector. Treatment of these cells with FTI-277 or FTI-2153 resulted in a concentration-dependent inhibition of oncogenic H-Ras activation of erk1 and erk2, which was blocked at 1 and 0.01μM, respectively (Fig. 3). Thus, FTI-2153 is 10-fold more potent than FTI-277 at suppressing oncogenic H-Ras activation of MAPK. The fact that suppression of MAPK activation is slightly more sensitive than inhibition of Ras processing suggests that either newly synthesized Ras or other farnesylated proteins are involved in FTI inhibition of MAPK activity.

FTI-2153 Is a More Potent Inhibitor of Human Tumor Growth in Soft Agar Than FTI-277. We next evaluated the ability of FTI-2153 and FTI-277 to inhibit the growth of a panel of human tumors in soft agar. Two pancreatic (Colo-357 and Panc-1), one lung adenocarcinoma (A-549), one astrocytoma (U87MG), and one medulloblastoma (DaOY) human cancer cell lines were used. Cells were plated in 12-well plates and treated with FTI-2153 or FTI-277 as described in "Materials and Methods." Fig. 4 shows that FTI-277 (25 μM) had little effect on the growth of Panc-1, Colo-357, and U87MG, whereas FTI-2153 at the same concentrations inhibited the growth of these cell lines in soft agar. Furthermore, the growth of A-549 cells was partially inhibited by FTI-277 at 25μM but was completely inhibited with FTI-2153 at 1 μM (Fig. 4). Similarly, whereas FTI-277 (5 μM) inhibited partially the growth of DaOY, FTI-2153 blocked the growth of this cell line at a 10-fold lower concentration (0.5 μM). Table 2 shows IC_{50}s of FTI-277 and FTI-2153 toward inhibiting different human tumor cell lines and further demonstrates that FTI-2153 is a more potent inhibitor of human tumor growth in soft agar. We have also found that oncogenic H-Ras-transformed NIH 3T3 cells are more sensitive than their parental cells to FTI-2153. FTI-2153 inhibited the growth on plastic of H-Ras-transformed NIH 3T3 cells and their parental cells with IC_{50}s of 0.3 and 10 μM, respectively. Furthermore,

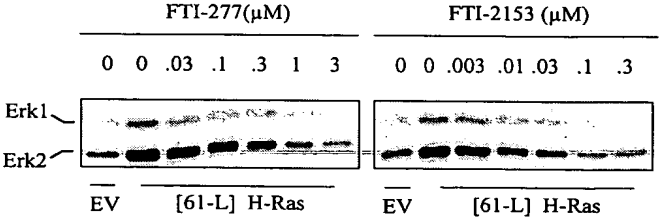

Fig. 3. Inhibition of oncogenic H-Ras activation of erk1 and erk2 by FTI-277 and FTI-2153. Oncogenic H-Ras-transformed NIH-3T3 cells were treated with the indicated concentrations of FTI-277 and FTI-2153 for 48 h, harvested and processed for SDS-PAGE, and immunoblotted with an antibody that recognizes specifically the phosphorylated forms of erk1 and erk2 as described in "Materials and Methods." Data are representative of three independent experiments. *EV*, empty vector.

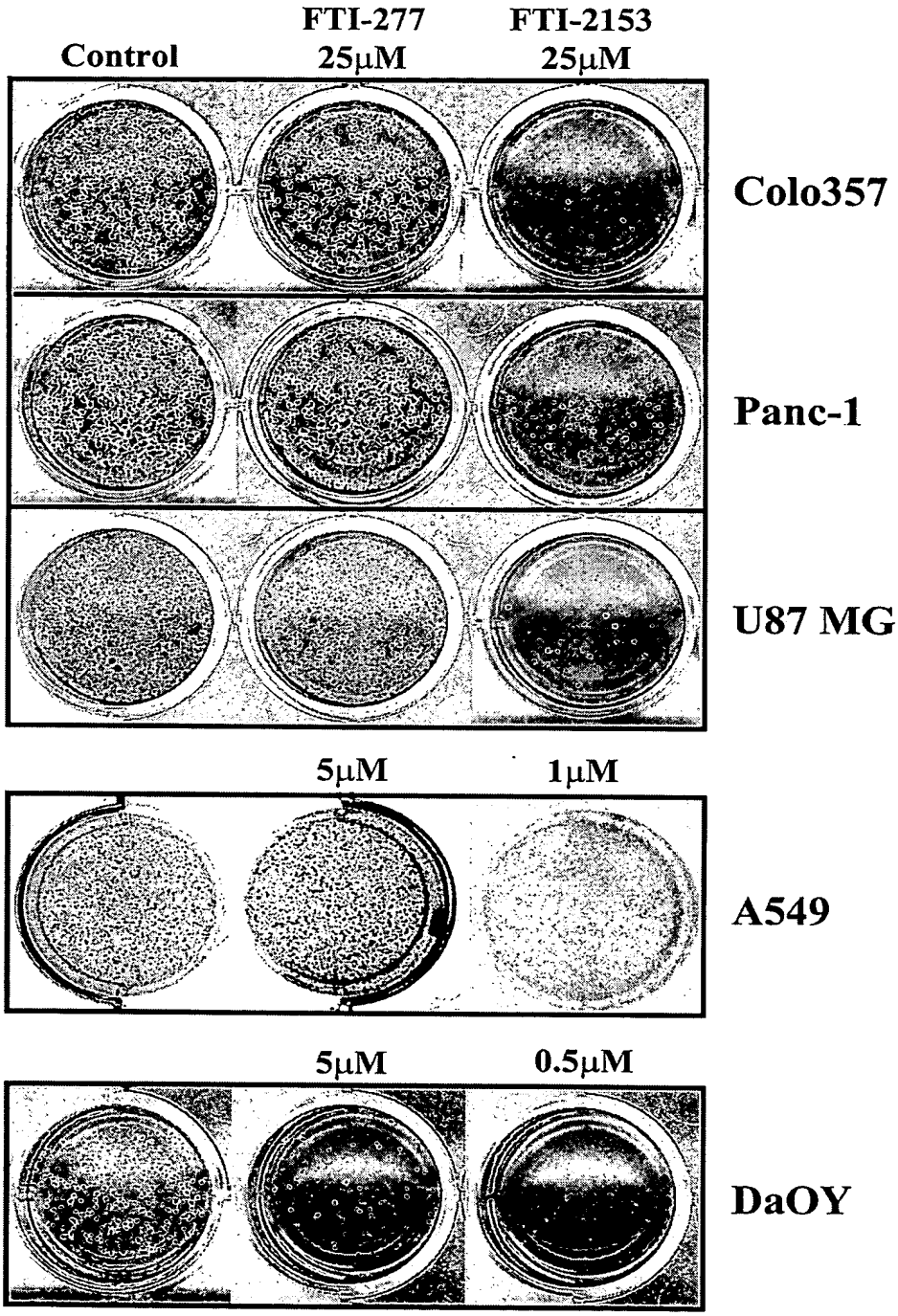

Fig. 4. Inhibition of soft agar growth of human tumor cells by FTI-277 and FTI-2153. Human pancreatic (Colo357 and Panc-1), lung adenocarcinoma (A-549), astrocytoma (U87-MG), and medulloblastoma (DaOY) cells were plated and treated with inhibitors as described in "Materials and Methods." The cells were then stained at the time indicated in "Materials and Methods" and photographed. Data are representative of two independent experiments. Each experiment was done in triplicate.

Table 2 *Effects of FTI-2153 on soft agar growth of various human tumor cells*

	IC_{50} (μM)	
Tumor line	FTI-277	FTI-2153
DaOY	5	0.1
A549	15	1
Colo357	>25	5
Panc-1	>25	5
U87-MG	>25	5

FTI-2153 at 30 μM resulted in 40% cell death (as measured by trypan blue staining) of H-Ras-transformed NIH 3T3 cells and only 7% of NIH 3T3 cells.

FTI-2148 Is a Better Antitumor Agent Than FTI-276 in the Human Xenograft Nude Mouse Model. Previously, we had demonstrated that FTI-276 delivered i.p. inhibits human tumor growth (12, 13). To evaluate the effects of replacing the reduced cysteine by an imidazole derivative and the phenyl group by a tolyl, we next compared the antitumor potency of FTI-2148 and FTI-276 (both i.p. and

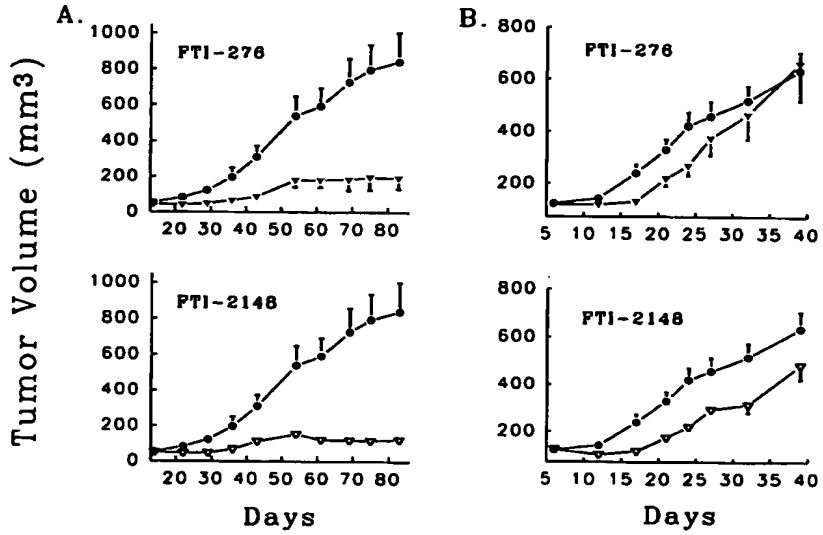

Fig. 5. FTI-2148 is a more potent antitumor agent than FTI-276. Human lung adenocarcinoma A-549 cells were implanted s.c. in nude mice, and when tumor sizes reached ~100 mm^3, animals were randomized. *A*, animals were treated i.p. with vehicle (●), FTI-276 (50 mpk/day; ▼), or FTI-2148 (50 mpk/day; ▽). At day 45, treatment was stopped, and at day 53, treatment was resumed. *B*, animals were s.c. implanted with 14-day mini-pumps filled with vehicle (●), FTI-276 (25 mpk/day; ▼), or FTI-2148 (25 mpk/day; ▽). Mice were followed an additional 2 weeks after drug cessation. Data are representative of two independent experiments. *Bars*, SE.

s.c. delivery) in the human lung adenocarcinoma A-549 cell nude mouse model. A-549 cells were implanted s.c. in nude mice, and when the tumors reached an average size of ~50–100 mm^3, the animals were randomized and treated either with vehicle or peptidomimetics as described in "Materials and Methods." Fig. 5*A* shows that, over a period of 83 days, tumors from animals that were treated with vehicle reached an average size of 839.56 ± 162.66 mm^3, whereas those treated with FTI-276 or FTI-2148 grew to average sizes of 193.21 ± 61.88 mm^3 or 122.95 ± 17.36 mm^3, respectively. Thus, FTI-276 and FTI-2148 inhibited A-549 tumor growth by 82 and 91%, respectively. In the above experiment, daily treatment (i.p.) was started on day 15 after s.c. implantation of tumors, stopped on day 45, and restarted on day 53 until day 83. Fig. 5*A* also shows that the tumors from the drug-treated animals grew faster when treatment was stopped, but that growth was repressed when treatment was resumed. This suggested that the effect of FTI-276 and FTI-2148 on A-549 tumor growth in nude mice is cytostatic. To confirm this, we treated A-549 cell-bearing nude mice with FTI-276 or FTI-2148 (25 mpk/day for 14 days) and followed tumor growth for an additional 15 days after drug cessation. Drugs were delivered by mini-pumps implanted s.c. in the opposite flank of tumor implants. Fig. 5*B* shows that FTI-276 and FTI-2148 delivered via s.c. mini-pumps at a rate of 25 mpk/day for 14 days inhibited tumor growth by 50 and 77%, respectively, by the end of the 2-week treatment. Furthermore, after drug treatment was stopped, the tumors from the FTI-276 group grew faster than those from the FTI-2148 group. Within 2 weeks of drug cessation, the tumors from the FTI-276-treated animals had grown rapidly and had reached the same average size as those from animals treated with vehicle (Fig. 5*B*). In contrast, tumors from the FTI-2148 treated animals grew slower, and even after 2 weeks of no drug treatment had an average size (480.81 ± 61.43 mm^3) 30% smaller than those from vehicle-treated controls (632.73 ± 69.67 mm^3). Thus, the data show that FTI-2148 is more effective than FTI-276. Furthermore, with both FTIs, the inhibition of A-549 tumor growth in nude mice was cytostatic and reversible.

GGTI-2154 Inhibits Human Tumor Growth in Nude Mice. We next determined whether the inhibitor of GGTase I, GGTI-2154, can inhibit human tumor growth in nude mice. Animals were implanted s.c. with A-549 cells and treated as described in "Materials and Methods." Fig. 6 shows that over a period of 52 days, tumors from vehicle-treated animals reached an average size of 608.21 ± 133.94 mm^3. In contrast, tumors from animals treated with either GGTI-297 or GGTI-2154 (50 mpk/day i.p.) had average tumor sizes of 356.35 ± 109.38 mm^3 and 287.90 ± 75.30 mm^3, respectively, over the same period of time (Fig. 6). Thus, GGTI-297 and GGTI-2154 inhibited A-549 tumor growth in nude mice by 47 and 60%, respectively.

Combination Therapy of FTI-2148 with Cisplatin, Gemcitabine, and Taxol Is More Efficacious than Monotherapy. Fig. 5 clearly demonstrates that FTI-2148 is a potent inhibitor of human tumor growth in nude mice but that when drug treatment was stopped, the tumors started to grow again. Thus, FTI-2148 exerts its antitumor activity via a cytostatic, reversible mechanism. We,

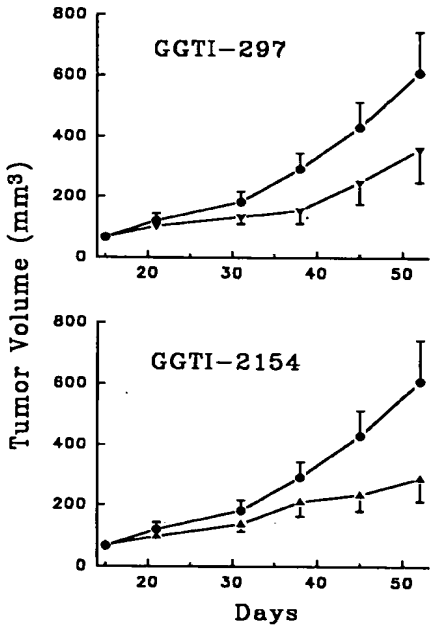

Fig. 6. Antitumor activity of GGTI-2154 and GGTI-297. A-549 cells were implanted as described above in the legend to Fig. 5. Animals were treated i.p. with vehicle ( ), GGTI-297 (50 mpk/day; ▼), or GGTI-2154 (50 mpk/day; ▲). Data are representative of two independent experiments. *Bars*, SE.

therefore, determined whether combining FTI-2148 with clinically used agents with different mechanisms of action would result in enhancing FTI-2148 antitumor activity. Fig. 7 shows that treatment with FTI-2148 (14 days, s.c., mini-pump at 25 mpk) resulted in 67% tumor growth inhibition ($P < 0.005$), whereas treatment with cisplatin (5 mpk, i.p., q4d, three times) resulted in 21% growth inhibition, which was not statistically significant from control ($P = 0.19$). Combination therapy of FTI-2148 and cisplatin (same doses and regimens) resulted in 85% ($P < 0.005$) tumor growth inhibition over the same period of time (Fig. 7*A*). Similarly, treatment with FTI-2148 (25 mpk, 14 days, s.c. mini-pumps) and gemcitabine (80 mpk, i.p., q4d, three times) resulted in 67% ($P < 0.005$) and 43% ($P = 0.09$), respectively, whereas combination therapy of these two agents resulted in 86% ($P < 0.005$) tumor growth inhibition (Fig. 7*B*). Finally, treatment with FTI-2148 (12.5 mpk, 14 days, s.c. mini-pumps) or Taxol (12.5 mpk, i.p., q4d, three times) resulted in 33% ($P = 0.07$) or 37% ($P < 0.05$) size reduction, respectively (Fig. 7*C*), whereas combination therapy resulted in 67% ($P < 0.005$) tumor growth inhibition (Fig. 7*C*). More importantly, tumors from animals treated with combination therapy (FTI-2148, cisplatin) took 43 days to reach 125 mm^3, whereas those from animals treated with cisplatin or FTI-2148 alone took only 25 and 30 days to reach the same tumor volume. Thus, combination therapy slowed down tumor growth by 40 and 30% as compared with monotherapy with cisplatin and FTI-2148, respectively. Similar results were obtained with gemcitabine and Taxol combination.

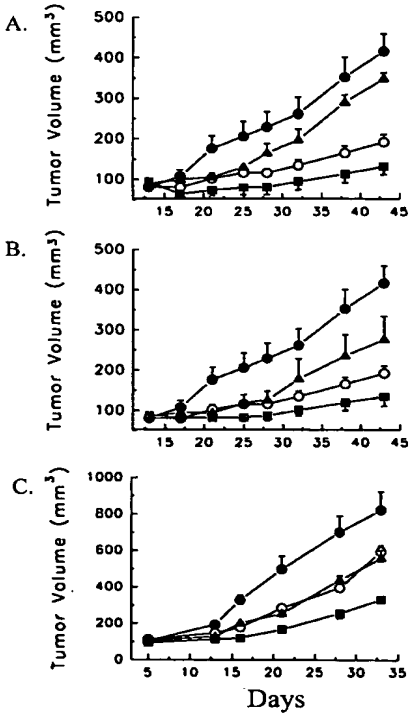

Fig. 7. Combination therapy of FTI-2148 with cisplatin, gemcitabine, and Taxol. A-549 cells were implanted s.c. under the left flank of nude mice, and when the tumor sizes reached ~100 mm^3, animals were randomized and implanted s.c. under the right flank with mini-pumps either filled with vehicle (●) and (▲) or FTI-2148 (○) and (■). Three days later, the animals were injected i.p. either with vehicle (●) and (○) or with cytotoxic agents (▲) and (■); *A*, cisplatin; *B*, gemcitabine; *C*, Taxol) as described in "Materials and Methods." Data are representative of two independent experiments. *Bars*, SE.

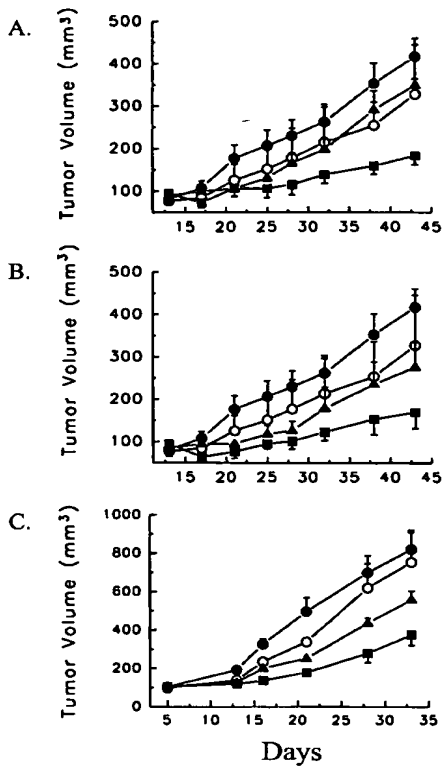

Fig. 8. Combination therapy with GGTI-2154 with cisplatin, gemcitabine, and Taxol. This experiment was carried out under identical conditions as those described in the legend to Fig. 7 except that GGTI-2154 and not FTI-2148 was used. Data are representative of two independent experiments. *Bars*, SE.

Combination Therapy of GGTI-2154 with Cisplatin, Gemcitabine, and Taxol Is More Efficacious than Monotherapy. Similar combination therapy experiments to those described above for FTI-2148 were also carried out for GGTI-2154 to determine whether this GGTase I inhibitor would also benefit from combination treatment with classic anticancer drugs. Fig. 8*A* shows that treatment with GGTI-2154 (25 mpk, 14 days, s.c., mini-pumps) or cisplatin (5 mpk, i.p., q4d, three times) resulted in 27 and 21% tumor growth inhibition, respectively. This tumor growth inhibition is not statistically significant ($P = 0.5$ and $P = 0.19$, respectively). Combination therapy of these two agents (same doses, same regimens) resulted in 71% tumor growth inhibition (Fig. 8*A*), which was statistically significant ($P < 0.005$). Similar experiments with FTI-2154 and gemcitabine (80 mpk, i.p., q4d, three times) in monotherapy gave 27% ($P = 0.5$) and 43% ($P = 0.09$) inhibition, and in combination therapy showed 63% ($P < 0.005$) tumor growth inhibition (Fig. 8*B*). Finally, monotherapy of GGTI-2154 (12.5 mpk, 14 days, s.c., mini-pumps) with Taxol (12.5 mpk, i.p. every third day, three times) resulted in 9% ($P = 0.7$) and 37% ($P < 0.05$) reduction, respectively, and in combination therapy in 60% ($P < 0.005$) tumor growth inhibition (Fig. 8*C*). Tumors from animals treated with combination therapy (GGTI-2154, cisplatin) took 43 days to reach 175 mm^3, whereas those from animals treated with cisplatin or GGTI-2154 alone took 27 days each to reach the same tumor volume. Thus, combination therapy greatly decreased tumor growth as compared with monotherapy. Similar results were obtained with the other two combinations. Furthermore, in each of the three cases, combination therapy slowed down tumor regrowth after drug cessation. At day 43, 2 weeks after drug

cessation, combination therapy had a statistically significant tumor growth inhibition.

DISCUSSION

The demonstration of potent inhibition of farnesyltransferase by tetrapeptides corresponding to the COOH terminal CAAX of Ras proteins prompted many to design peptidomimetics to overcome cellular uptake and peptidase degradation problems of these tetrapeptides. To this end, we have taken a systematic approach to the replacement of the three major portions of the tetrapeptide. Previously, we have reported on the design and biological activity of CAAX peptidomimetics where the central hydrophobic portion "AA" and the COOH terminal tripeptide "AAX" were replaced by di- and tripeptide mimetics, respectively (5, 6, 24). All of these molecules contain the oxidizable cysteine thiol group, which is highly reactive and could result in undesirable pharmacokinetic as well as pharmacodynamic effects that would hamper the development of these peptidomimetics as anticancer drugs. In this report, we describe the design of non-thiol peptidomimetics where "CAA" was replaced by an imidazole derivative of 2-(2-methylphenyl)-4-aminomethylbenzoic acid. The resulting peptidomimetic FTI-2148 (IC_{50}, 1.4 nM) is much more potent than the parent tetrapeptide CVIM (IC_{50}, 200 nM; ref. 26) and is highly selective (1200-fold) for FTase over the closely related family member GGTase I. Furthermore, although FTI-2148 was slightly less potent *in vitro* than FTI-276, in whole cells, its methyl ester prodrug FTI-2153 (IC_{50}, 0.01 μM) was much more potent than the corresponding FTI-277 (IC_{50}, 0.3 μM). This increase in whole-cell potency may be due to a greater cellular uptake because of the more hydrophobic character of the tolyl and the imidazole groups relative to the phenyl and cysteine groups. Hunt *et al.* (27) also replaced a cysteine by imidazole and prepared a CAAX peptidomimetic with an *in vitro* FTase IC_{50} of 0.79 nM, but its ability to inhibit H-Ras processing in whole cells was much less potent (3.8 μM). Finally, the non-thiol-containing FTI-2148 and FTI-2153 molecules present another important advantage over FTI-276 and FTI-277 because of their high selectivity for FTase over GGTase I. Indeed, FTI-2148 is 1200-fold, whereas FTI-276 is only 100-fold, more selective *in vitro*. Similarly, in whole cells, FTI-2153 is >3000-fold, whereas FTI-277 is only >100-fold, more selective.

The effects of the tolyl and imidazole replacements on selectivity is even more pronounced for GGTase I inhibitors (28). *In vitro*, GGTI-297 is only 4-fold, whereas GGTI-2154 is 266-fold, more selective for GGTase I over FTase. Similarly, in whole cells, GGTI-298 is only >3-fold, whereas GGTI-2166 is >100-fold, more selective. These findings have important implications for further biological work exploring physiological consequences of selective inhibition of protein geranylgeranylation. It is also important to point out that at concentrations where GGTI-298 inhibition of Rap1A processing is complete, this agent becomes toxic, whereas GGTI-2166 concentrations that block Rap1A processing are not toxic (data not shown). The ability of FTI-2153 to block oncogenic H-Ras activation of MAPK as well as its anti-transforming activity against human tumors grown in soft agar was also superior when compared with FTI-277. Furthermore, after drug removal, the A-549 tumors from FTI-2148-treated animals grew more slowly than those from FTI-276-treated animals. Chronic treatment over a period of almost 3 months resulted in no gross toxicity. This lack of FTI toxicity is consistent with previous reports (12–16).

Recently, we have shown that GGTI-297 inhibits human tumor growth in nude mice (12). However, because GGTI-297 is only 4-fold more selective for GGTase I over FTase, involvement of inhibition of farnesylation in the mechanism of antitumor activity of GGTI-297 is possible. In this report, we demonstrate that GGTI-2154, which is 266-fold more selective for GGTase I over FTase, is a more potent antitumor agent than GGTI-297, suggesting that the involvement of inhibition of protein farnesylation is unlikely.

Although FTI-2148 alone was capable of inhibiting human tumor growth, drug removal resulted in tumor regrowth. These cytostatic effects are not unique to FTI-2148 and have also been seen previously with other FTIs (14–16). Even in immunocompetent H-Ras transgenic mice where FTIs were shown to induce tumor regression, removal of drug still resulted in tumor regrowth (15). Because of this, it is believed that, clinically, the best use of FTIs as anticancer drugs might be in combination with cytotoxic agents. We, therefore, evaluated the effectiveness of combination therapy with cisplatin, gemcitabine, and Taxol. Our studies clearly demonstrate that combination therapy results in improved antitumor activity. Moreover, after drug removal, tumors from combination therapy groups grew very little as compared with those from monotherapy groups. A recent study (29) in cultured tumor cells showed that when FTIs are combined with cytotoxic agents that prevent microtubule depolymerization, such as Taxol or epothilones, a synergistic interaction resulted in inhibition of cell growth. This study suggested that *in vitro* inhibition of protein farnesylation results in enhanced mitotic sensitivity to Taxol. Our studies in whole animals show that combination therapy is additive rather than synergistic.

Thus, we have designed non-thiol-containing FTase and GGTase I inhibitors that are much more selective than our previously reported CAAX peptidomimetics. Not only are these agents more selective, but they also have superior antitumor activity while still lacking toxicity. Our results also demonstrate that combination therapy of these novel prenyltransferase inhibitors with cytotoxic agents results in improved antitumor activity. This is an important finding that has critical implications because FTIs are presently being evaluated clinically.

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